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Full Papers

A new method for the large scale preparation of antitoxic antibodies exhibiting high specific protective activities[1]

B. Favreau, D. Giurgiu and B. Bizzini[2]

Institut Pasteur, Département de Biochimie et Génétique Moléculaire, 28, rue du Dr. Roux, F-75724 Paris Cedex 15 (France), December 3, 1982

Summary. A method using polyethylene-glycol and immobilized pepsin for purifying heterologous antitoxic antibodies is described. Using horse antitetanus plasma or sera, $F(ab')_2$ fragments exhibiting specific activities in the range of 150 IU per mg protein were repeatedly isolated with a yield around 80%. The procedure was scaled up from 200 ml up to 20 l.

Introduction

Heterologous antitoxic sera have long been used for the treatment of toxi-infections: diphtheria and tetanus, as well as snake and scorpion envenoming. With the purpose of eliminating the risk of adverse reactions, whole sera have been subjected to pepsin digestion which results in the destruction of the Fc fragment responsible for the reactogenicity of the

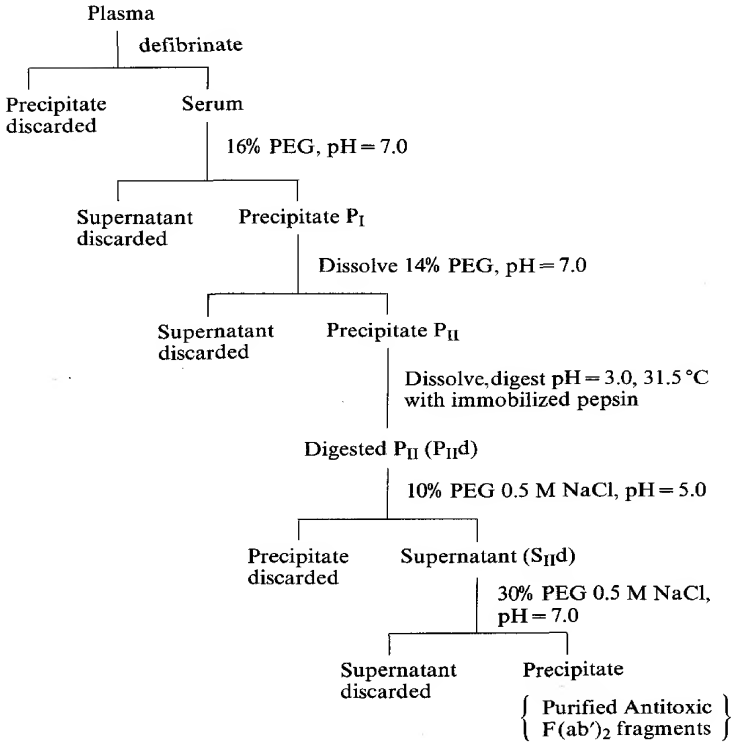
antibody molecule. The remaining $F(ab')_2$ portion of the antibody molecule has further been purified by ammonium sulfate fractionation. Purified digested heterologous sera proved to be more readily tolerated than crude whole sera. More recently, homologous sera have been substituted for heterologous sera, at least in developed countries. However, heterologous sera are and will continue to be widely used in developing countries because of the practically unlimited supply of such sera and because of their low cost.

Up to now, the universally followed industrial procedure for purifying antitoxic sera has been based on the method described by Parfentjev[17] and modified by Pope[21,22]. It is a rather empirical and time-consuming procedure. The yield in purified antitoxic $F(ab')_2$ fragments is variable, but it does not exceed 50%.

Therefore, it has appeared to us that studying a new method for purifying antitoxic antibodies on a large scale would be worthwhile. The method that we have developed consists of 3 main stages. The 1st step uses the polyethylene-glycol (PEG) as a precipitating agent to isolate the whole immunoglobulin fraction (Ig fraction) from the serum. In the 2nd step the Ig fraction is digested by pepsin coupled to porous glass beads. The 3rd step uses PEG to precipitate the $F(ab')_2$ fraction generated by the pepsin digestion of the Ig fraction.

The results reported here were obtained when this procedure was applied to the purification of horse antitetanus antisera either on a small scale (200–2000 ml) or on a larger scale (20 l).

Schematic description of the purification procedure of heterologous antitetanus antisera.



Materials and methods

The antitetanus antibody source consisted of either pooled antitetanus plasma or sera from hyperimmunized horses (Institut Pasteur Production, Marnes-la-Coquette, France). The chemicals were of the best available quality (Sigma, Saint Louis, USA). Porous silica beads (CPG A1100) grafted with an arm carrying a terminal NH_2 group were a gift from Corning Glass Europe, Fontainebleau, France. Anti-horse IgG and anti-horse albumin sera were from Nordic, Tilburg, The Netherlands. Anti-horse F_c fragment was a gift from Dr Iscaki (Institut Pasteur, Paris, France). Anti-whole horse serum was raised in rabbits in our laboratory.

Protein determinations were according to Lowry et al.[14] or according to Mancini et al.[15] by radial immunodiffusion. Protein nitrogen contents were determined by the microkjeldahl method[16].

The antitetanus activity of sera or serum fractions was assayed either by flocculation and expressed as Lf units[23], or by radial immunodiffusion (Lf units), or by the mouse in vivo test and expressed as IU[26].

The purification was followed by immuno-electrophoresis[10]. The presence of aggregated forms in purified $F(ab')_2$ preparations was monitored by gel filtration on Sephadex G 200 and Sepharose 4B. Polyethyleneglycol solutions were prepared by dissolving PEG_{6000} in the appropriate buffer to a concentration twice that attained in the reaction mixture for precipitating a particular protein. The pH of the solutions was checked after complete dissolution of PEG_{6000}.

Pepsin was immobilized by coupling to aminated porous glass beads (CPG A1100). Coupling was carried out as follows: 20 g of glass beads (bed volume: 70 ml) were thoroughly washed first with distilled water and then with 4 volumes of 0.1 M bicarbonate buffer pH 8.0. The washing process was repeated once more and the glass beads were equilibrated at pH 4.5 with saline adjusted to pH 4.5 by the addition of 0.1 M acetate buffer. The beads were mixed with 56 ml of a solution containing 3.5 g of pepsin (2 × cryst. lyophilized hog stomach pepsin: Sigma) and 14 ml of a solution containing 2.1 g of carbodiimide (1-cyclohexyl-3 (2-morpholinoethyl) carbodiimide meto-p-toluene sulfonate). The pH of the reaction mixture was maintained at 4.5 during the 1st h of the reaction by addition of either 0.1 N HCl or 0.1 N NaOH. The reaction mixture was subsequently allowed to stand for 20 h at 4 °C under gentle stirring. The reaction was terminated by washing the glass beads on a fritted glass funnel with saline pH 4.5 and 0.06 N HCl successively. The beads were finally equilibrated by filtering through them an adequate

volume of 0.1 M acetate buffer pH 4.5 and stored at 4 °C in the same buffer.

The purification procedure devised for purifying antitetanus antibodies is schematically depicted in the figure.

When plasma was used, it was first defibrinated at 37 °C by the addition of 20 ml of 20% $CaCl_2$ solution per liter of plasma. The plasma was vigourously stirred with a cooking whip for about 5–10 min and then stirred intermittently until the end of the reaction. The fibrin clot forms in long filaments around the whip. Clotting was complete within 2–3 h. When old plasma is used, exogenous thrombin (Thrombase 500: Lab. Houdé, Paris, France) may have to be added (1500 units per l of plasma) to promote clotting. We also devised an alternative method for defibrinating the plasma which consists of adding to 1 volume of plasma at pH 8.0, 1 volume of 8% PEG_{6000} solution, while stirring. Under these conditions, fibrinogen is quantitatively precipitated.

The whole Ig fraction can be isolated from the serum or defibrinated plasma by 2 successive precipitations with PEG_{6000}. The first precipitation is carried out by the addition, by drop, of 1 volume of a 32% PEG_{6000} solution to 1 volume of serum at pH 7.0 while stirring. After the addition of PEG, the solution is allowed to stand at room temperature for 30–45 min and the precipitate (P_I) that has formed is recovered by either centrifugation (12,000 × g for 10 min) when working with small volumes (up to 200 ml) or filtration through a 50-µm nylon mesh cloth when working with large volumes. The precipitate is redissolved in a volume of saline equal to ¾ of the final volume of the first precipitation. The pH of this solution is adjusted to 7.0 and the Ig fraction is precipitated by adding an equal volume of 28% PEG_{6000} solution. The thus formed precipitate (P_{II}) is dissolved in a volume of saline corresponding to that of the solution of P_I. The pH is adjusted to 3.0 with 1 N HCl and the solution is heated to 31.5 °C in either a thermostated water-bath (small volumes) or in a digestor equipped with double walls for the circulation of fluid at the appropriate temperature (large volumes). The digestion is carried out for 90 min using 200 activity units[3], or 5 mg of the particular preparation of pepsin coupled to glass beads, per ml of P_{II} solution. We terminated digestion by filtering off the insolubilized pepsin and adjusting the pH of the filtrate to 7.5 with 1 N NaOH, while stirring. An opalescence may form as a result of the pH adjustment, but this does not interfere with the subsequent steps of the purification procedure. The solution of the digested P_{II} ($P_{II}d$) contains predominantly $F(ab')_2$ fragments and a low amount of contaminants consisting of aggregates of $F(ab')_2$ fragments as well as of low-molecular-weight polypeptides. The bulk of the contaminants can be precipitated from the $P_{II}d$ solution at pH 5.0 by the addition of PEG_{6000} to the final concentration of 10% in the presence of 0.5 M NaCl. The $F(ab')_2$ fragments remain in solution. The precipitate is filtered off and after adjustment of the filtrate to pH 7.0, the $F(ab')_2$ fragments are precipitated to a final PEG_{6000} concentration of 30%.

The precipitate of $F(ab')_2$ fragments is redissolved in the minimum volume of neutral saline and it is dialyzed against a large volume of saline.

Results and discussion

The procedure schematically represented in the figure for the purification of antitetanus antibodies uses the capacity of polyethylene-glycol to selectively precipitate proteins under mild and non-denaturing conditions. Polson et al.[20] first systematically studied the selective precipitation of serum proteins by means of PEG. The results of these studies have subsequently been furthered by other investigators[8,9,13,19].

The results reported in the table show that precipitation of the serum at pH 7.0 to final PEG_{6000} concentrations of 16% and 14% permits the isolation, in successive stages, of the Ig fraction (P_{II}) from the serum with a high yield, since P_{II} contains more than 80% of the original antitetanus activity of the serum. The fraction is still contaminated with a small quantity of albumin (5%) as revealed by immuno-electrophoresis and quantitated by radial immuno-diffusion. At this stage of purification the specific protective activity of P_{II} is close to 60 IU per mg protein.

In order for us to better control the digestion of the purified Ig fraction (P_{II}), we carried it out with pepsin immobilized by coupling to porous glass beads. Pepsin coupled to AH Sepharose has previously been

Results of the purification of a 2-l sample of horse antitetanus plasma

Products	Percent precipitated in reference to the				Specific activities	
	Proteins	Ig*	Ig-AT**	Albumin	Lf/mg protein	UI/mg protein
Serum	100	100	100	100	27	40
P_I	75	96	93	30	36	52
P_{II}	64	87	84	5	39	61
P_{IId}	64	-	84	Traces	-	-
$F(ab')_2$	26	-	79	0	104	150

*Ig: whole immunoglobulin fraction; **Ig-AT: immunoglobin fraction with antitetanus activity.

used for digesting immunoglobulins[4,25]. The main advantage of using glass beads instead of Sepharose gel is the definitely better mechanical resistance of the former and also the likelihood that more enzyme molecules would bind to glass beads than to derivatized Sepharose. Furthermore, digestion can be interrupted easily by removing the immobilized enzyme from the reaction mixture which can be used for further digestion cycles.

The results reported in the table also show that the Ig fraction (P_{II}) can readily be digested without loss of antitetanus activity to yield fraction $P_{II}d$. In fact, $P_{II}d$ exhibits the same antitetanus activity as P_{II}. Furthermore, $P_{II}d$ contains only trace amounts of albumin. Pepsin digestion of the Ig fraction (P_{II}) results in the formation of $F(ab')_2$ fragments, as well as some aggregated $F(ab')_2$ and of small-molecular-weight compounds. However, the aggregation can be reduced to a minimum by carrying out the digestion in the presence of 0.22 M glycine, at pH 3.0, and by adjusting the concentration of the solution to 0.5 M NaCl after digestion and before neutralization. If present, the small quantities of aggregated $F(ab')_2$ fragments can be eliminated by precipitating the $P_{II}d$ solution at pH 5.0 to a final PEG_{6000} concentration of 10% in the presence of 0.5 M NaCl. $F(ab')_2$ fragments and low-molecular-weight products remain in solution. After removing aggregated $F(ab')_2$ fragments by filtration, the $F(ab')_2$ fragments are selectively precipitated from the filtrate at pH 7.0 to a final PEG concentration of 30%, whereas low-molecular-weight compounds remain in solution together with most of the PEG. The last traces of PEG contaminating the $F(ab')_2$ fraction can be eliminated from the solution of this fraction, if required, by ultrafiltration using hollow fibers with a breakthrough point of 50,000 daltons. Busby and Ingham[7] have similarly removed PEG from albumin solutions.

A $F(ab')_2$ fraction is thus obtained which contains 79% of the antitetanus activity originally present in the serum. The specific protective activity of these $F(ab')_2$ fragments is high: 150 IU per mg protein (table). The absence of the Fc fragment in the preparation of $F(ab')_2$ fragments was checked by double gel diffusion and immuno-electrophoresis using a sheep anti-horse Fc serum.

The specific antitetanus protective activity of $F(ab')_2$ fragments obtained by application of the purification procedure described in the present paper (around 150 IU per mg protein) compares advantageously with activities (ranging from 60 to 117 IU per mg protein) reported by other investigators using immunoaffinity techniques to isolate monospecific antitetanus antibodies[5,18,24,27]. In addition, when we isolated by immunoaffinity chromatography from the $F(ab')_2$ fragments purified by the present method the antitetanus directed fraction, we obtained a fraction exhibiting a sp. act. of about 450 IU per mg protein. Relyveld (personal communication), in using the immunopurification procedure he describes for separating the monospecific antitetanus antibodies from the same $F(ab')_2$ fraction as above, determined a sp.act. of 355 IU per mg protein[24].

Following the method of Iscaki and Raynaud[12], as modified by Iscaki[11], equine antitetanus F(ab') fragments have been prepared from $F(ab')_2$ fragments purified as described in the present paper. F(ab') preparations obtained in this way exhibited specific activities in the range of 130–150 IU per mg protein. F(ab') fragments demonstrated a capacity to displace and neutralize tetanus toxin bound to synaptic membranes isolated from the rat spinal cord[6]. The F(ab') fragments also proved to be quite effective by the intrathecal route in the treatment of human tetanus, since their use resulted in the drastic decrease in mortality. They were also found to be devoid of undesirable effects[28].

In conclusion, it is worth mentioning that similar results have been obtained when a great deal of horse antitetanus plasma of a volume varying from 200 ml up to 20 l was purified by the present method. This procedure could profitably be used in conjunction with immunopurification methods for the purpose of preparing monospecific antitoxic antibodies of very high specific activities.

Since the usual Parfentjev-Pope method applies with minor modifications to the purification of antitoxic sera of different specificities, it is safe to predict that this will also hold true for the method described in the present paper.

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2 To whom correspondence should be addressed.

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Short Communications

Enzymatic preparation of [U-^{14}C]-4-chloronitrosobenzene[1]

M.D. Corbett and B.R. Corbett

Pesticide Research Laboratory, Food Science and Human Nutrition Department, University of Florida, Gainesville (Florida 32611, USA), August 3, 1982

Summary. [U-^{14}C]-4-Chloroaniline (**1**) was converted in good yield to [U-^{14}C]-4-chloronitrosobenzene (**3**) by oxidation with H_2O_2 in the presence of chloroperoxidase.

In recent years, aromatic C-nitroso compounds have been a subject of considerable interest to biochemical toxicologists. Mammalian metabolism of aromatic amines and nitro compounds is known in many cases to produce the nitroso oxidation state as an intermediary metabolite[2]. The toxicity of aromatic amine and nitro compounds, including mutagenic properties, is probably the result of metabolic production of the nitroso and similar reactive metabolites. In our own work we have found that nitroso aromatics will react under physiological conditions to produce hydroxamic acids[3,4], which are also known to be toxic metabolites. A convenient route to radiolabeled nitroso compounds of high specific activity and purity was necessary to continue our studies. We now report the development of such a method, which is based on a previously-reported enzymatic oxidation.

We could find no literature precedence for the preparation of ^{14}C-labeled nitroso aromatic compounds. The most general procedure for the synthesis of aryl nitroso compounds involves reduction of the corresponding nitro compound selectively to the hydroxylamine oxidation state, followed by mild oxidation of the hydroxylamine to the nitroso compound[5]. ^{15}N-Labeled nitrosobenzene was synthesized in 38% yield by such a procedure; however, the sequence employed 16 mmoles of starting material[6]. It is our experience that such a reduction-oxidation sequence is not adaptable to the preparation of nitroso compounds with high specific activity from μmole quantities of starting material. A second but less common method for the synthesis of aryl nitroso compounds is the direct oxidation of an arylamine to the nitroso compound by use of peracids[7]. This chemical oxidation generally results in poor yields with the azo, azoxy and nitro compounds being major contaminants.

The unique ability of the fungal enzyme, chloroperoxidase [E.C. 1.11.1.10], to catalyze peroxide oxidation of arylamines to the nitroso oxidation state was previously described by us[8,9]. This enzymatic oxidation proceeds through initial production of the arylhydroxylamine (e.g. **2**), which in most cases is more rapidly oxidized to the nitroso compound than is the starting arylamine. We have found that this enzymatic oxidation can be scaled-up to the degree that makes it useful for μmolar preparative reactions. The only labeled substrate investigated was [U-^{14}C]-4-chloroaniline (**1**); however, prior studies with unlabeled arylamine substrates indicate that the method should be of general value[9], except for those arylamines possessing considerable steric hindrance about the amine functional group[8].

The general method involves incubation of enzyme, H_2O_2 and the substrate arylamine at a concentration consistent with its solubility in aqueous buffer (generally < 0.5 mM). Only EtOH was investigated as a solvent for addition of the